



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/57, 1/21, 9/56, 15/75, 15/62	A2	(11) International Publication Number: WO 97/39130 (43) International Publication Date: 23 October 1997 (23.10.97)
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(21) International Application Number: PCT/US97/06477

(22) International Filing Date: 16 April 1997 (16.04.97)

(30) Priority Data:
08/634,440 18 April 1996 (18.04.96) US

(60) Parent Application or Grant

(63) Related by Continuation
US 08/634,440 (CON)
Filed on 18 April 1996 (18.04.96)

(71) Applicants (for all designated States except US): NORTH CAROLINA STATE UNIVERSITY [US/US]; 103 Holaday Hall, Campus Box 7003, Raleigh, NC 27695-7003 (US). UNIVERSITY TECHNOLOGIES INTERNATIONAL, INC. [CA/CA]; 609 14th Street, N.W., Calgary, Alberta (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): SHIH, Jason, C., H. [US/US]; 100 Planters Wood Lane, Cary, NC 27511 (US). LIN, Xiang [CN/US]; Apartment 203, 3608 Horton Street, Raleigh, NC 27607 (US). WONG, Sui-Lam [CA/CA]; 38 Ranch Glen Drive, N.W., Calgary, Alberta T3G 1E3 (CA).

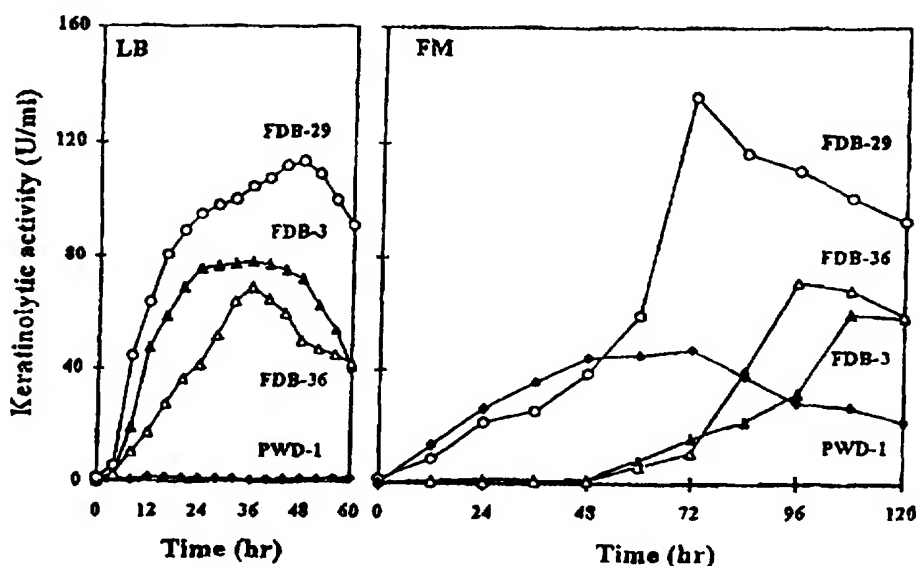
(74) Agents: SIBLEY, Kenneth, D. et al.; Myers, Bigel, Sibley & Sajovec, L.L.P., P.O. Box 37428, Raleigh, NC 27627 (US).

(81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD FOR EXPRESSING AND SECRETING KERATINASE



(57) Abstract

The present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. The present invention also provides a method for producing keratinase enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (b) collecting keratinase enzyme from the cell culture.

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METHOD FOR EXPRESSING AND SECRETING KERATINASE

This invention was made with Government support under grant number NRI-93-37500-9247 from the United States Department of Agriculture. The government has certain rights to this invention.

5 Field of the Invention

The present invention relates to cloning and expression of enzymes in and secretion by host cells, and in particular to cloning, expression, and secretion of keratinase in host cells.

10 Background of the Invention

Feathers are produced in large quantities by the poultry industry. These feathers provide an inexpensive source of raw material for a variety of potential uses. Among other things, there has been
15 considerable interest in developing methods of degrading feathers so they can be used as an inexpensive source of amino acids and digestible protein in animal feed. Processes for converting feather into animal feed which have been developed to
20 date include both steam hydrolysis processes and combined steam hydrolysis and enzymatic processes. See, e.g., Papadopoulos, M.C., *Animal Feed Science and Technology* **16**:151 (1986); Papadopoulos, M.C., *Poultry Science* **64**:1729 (1985); Alderibigde, A.O. et al., *J. Animal Science* **119**8 (1983); Thomas and Beeson, *J. Animal Science* **45**:819 (1977); Morris et al., *Poultry Science* **52**:858 (1973); Moran et al., *Poultry Science* **46**:456 (1967); Davis et al., *Processing of poultry by-products and their utilization in feeds, Part I, USDA Util. Res. Rep. no. 3, Washington, D.C. (1961).*
30 Disadvantages of these procedures, such as the

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degradation of heat sensitive amino acids by steam processes and the relatively low digestibility of the resulting products, have lead to continued interest in economical new feather degradation procedures which do not require a harsh steam treatment.

Keratinase enzyme has been found to be an effective feather degrading enzyme useful for converting keratin into amino acids for inclusion into animal feeds. U.S. Patent Application Serial No. 08/250,028 filed 27 May 1994 discloses an isolated *Bacillus licheniformis* PWD-1 keratinase enzyme for such use.

It is an object of the present invention to provide new, economical methods of producing keratinase.

It is a further object of the present invention to provide a host cell, and expression and secretion system for keratinase, which is capable of the hyperproduction of keratinase.

It is a further object of the present invention to provide recombinant DNA, host cells, and an expression and secretion system capable of hyperexpressing an enzyme encoded by a heterologous DNA.

Summary of the Invention

The foregoing objects are met by the present invention. As a first aspect, the present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. *Bacillus licheniformis* PWD-1 keratinase enzyme has the sequence as set forth in SEQ ID NO:1. In the preferred embodiment, the vector DNA further comprises a kera

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pre/pro processing and secretion region at nucleotides 215 through 529 of the keratinase gene (SEQ ID NO:1).

As a second aspect, the present invention provides a method for producing keratinase enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (b) collecting keratinase enzyme from the cell culture.

As a third aspect, the present invention provides an expression and secretion system for keratinase enzyme. The expression and secretion system includes (a) a *Bacillus subtilis* host cell, and (b) a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith.

As a fourth aspect, the present invention provides a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. The heterologous DNA encoding the enzyme may be a heterologous DNA encoding a proteinase, in particular a keratinase.

As a fifth aspect, the present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting an enzyme encoded by a heterologous DNA. The host cell contains a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

As a sixth aspect, the present invention provides a method of producing an enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis*

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host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, and (b) collecting enzyme from the
5 *Bacillus subtilis* host cell culture. Preferably, the heterologous DNA is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

As a seventh aspect, the present invention provides an expression and secretion system for an
10 enzyme. The system includes (a) a *Bacillus subtilis* host cell, and (b) a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme. Preferably, the heterologous DNA
15 is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme.

The foregoing and other objects and aspects of the present invention are explained in detail in the detailed description set forth below.

20 **Brief Description of the Drawings**

Figure 1 illustrates the construction of a plasmid, pLB3, containing the 1.45 kilobase *kerA* keratinase gene. *Km^r* denotes the kanamycin resistance gene.

25 **Figure 2** illustrates the structures of plasmids, pLB3, pLB29, and pLB36 all containing the 1.45 kilobase *kerA* keratinase gene. P43 represents the ~300 base pair fragment containing the vegetative growth promoter. *Km^r* denotes the kanamycin resistance
30 gene. Arrows indicate the orientations of genes.

Figure 3 illustrates the detection of proteolytic activity by formation of hydrolysis haloes on milk-agar plates. Plate A represents cell-free culture supernatants from 72-hour feather medium.
35 Plate B represents 36-hour cell-free culture supernatants from Luria-Bertani medium. The numbers on

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the plates represent culture supernatants from (1) PWD-1, (2) FDB-3, (3) FDB-29, (4) FDB-36, and (5) DB104/PUB18.

Figure 4 is a graphical illustration of the expression of *kerA* in FDB-3, FDB-29, and FDB-36 in Luria-Bertani (LB) medium and feather medium (FM). Keratinolytic activity was measured by azokeratin hydrolysis.

Figure 5 illustrates the immuno-diffusion assay of keratinase produced in culture media using rabbit anti-keratinase serum. Plate A contains cell-free culture supernatants from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 samples were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium. The numbers on plates represent culture supernatants from (1) PWD-1, (2) FDB-3, (3) FDB-29, (4) FDB-36, and (5) DB104/PUB18.

Figures 6A, 6B, and 6C are graphical illustrations of the effects of kanamycin on *kerA* expression. **Figure 6A** represents results obtained from bacterial strain FDB-3 in Luria-Bertani (LB) medium and feather medium (FM). **Figure 6B** represents results obtained from bacterial strain FDB-29 in Luria-Bertani (LB) medium and feather medium (FM). **Figure 6C** represents results obtained from bacterial strain FDB-36 in Luria-Bertani (LB) medium and feather medium (FM).

Detailed Description of the Invention

Amino acid sequences disclosed herein are presented in the amino to carboxy direction, from left to right. The amino and carboxy groups are not presented in the sequence. Nucleotide sequences are presented herein by single coding strand only, in the 5' to 3' direction, from left to right. Nucleotides

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and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by three letter code, in accordance with 37 CFR §1.822 and established usage.

5 See, e.g., PatentIn User Manual, 99-102 (Nov. 1990) (U.S. Patent and Trademark Office, Office of the Assistance Commissioner for Patents, Washington, D.C. 20231); U.S. Patent No. 4,871,670 to Hudson et al., at Col. 3, lines 2--43 (applicants specifically intend
10 that the disclosure of this and all other patent references cited herein be incorporated herein by reference).

A. DNA Encoding Keratinase Enzyme

DNA molecules which encode a keratinase
15 enzyme are those which encode a protein capable of degrading a keratin source such as feathers. This definition is intended to encompass natural allelic variations in the DNA molecules. As used herein, "natural" or "native" DNA refers to sequences isolated
20 from natural sources, as opposed to sequences created by chemical synthesis and not found in nature.

Hybridization conditions which will permit other DNA sequences which code on expression for a keratinase to hybridize to a DNA sequence as given
25 herein are, in general, high stringency conditions. For example, hybridization of such sequences may be carried out under conditions represented by a wash stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA disclosed herein in a
30 standard *in situ* hybridization assay. See, J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd. Ed. 1989) (Cold Spring Harbor Laboratory)). In general, DNA sequences which code for a keratinase and hybridize to the DNA sequence encoding the *Bacillus*
35 *licheniformis* PWD-1 keratinase disclosed herein will be at least 65%, 70%, 75%, 80%, 85%, 90%, or even 95%

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homologous or more with the sequence of the keratinase DNA disclosed herein.

Further, DNA sequences (or oligonucleotides) which code for the same keratinase as coded for by the foregoing sequences, but which differ in codon sequence from these due to the degeneracy of the genetic code, are also an aspect of this invention. The degeneracy of the genetic code, which allows difference nucleic acid sequences to code for the same protein or peptide, is well known in the literature. See, e.g., U.S. Patent No. 4,757,006 to Toole et al. at Col. 2, Table 1.

DNA sequences (or oligonucleotides) which code for the same keratinase as coded for by the foregoing sequences, but which differ in codon sequence from these due to site directed mutagenesis are also contemplated by this invention. Site directed mutagenesis techniques useful for improving the properties of the keratinase enzyme are well known, as described below. See, e.g., U.S. Patent No. 4,9873,192 to Kunkel.

As used herein, "kerA" refers to the 1.457 kilobase keratinase gene encoding keratinase and including the kerA pre/pro processing and secretion region. The nucleotide sequence for kerA gene is set forth in SEQ ID NO.:1. The amino acid sequence encoded by kerA is set forth in SEQ ID NO.:2. Also as used herein, "kerA pre/pro processing and secretion region" refers to the nucleotide sequence from nucleotide 215 to nucleotide 529 of the kerA gene, which comprises the pre-region (nucleotides 215-301) and the pro-region (nucleotides 302-529). The processing and secretion region of keratinase permit the cleavage and the extra-cellular secretion of the expressed protein. The pre-region of kerA encodes a signal peptide for secretion of the protein. The pro-region of kerA encodes a signal peptide which controls correct folding of the

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peptide. The mature protein of *kerA* extends from nucleotide 530 to nucleotide 1351, and encodes the 274 amino acid keratinase.

B. Genetic Engineering Techniques

5 The production of cloned genes, recombinant DNA, vectors, transformed host cells, proteins and protein fragments by genetic engineering is well known. See, e.g., U.S. Patent No. 4,761,371 to Bell et al. at Col. 6 line 3 to Col. 9, line 65; U.S. Patent No. 10 4,877,729 to Clark et al. at Col. 4, line 38 to Col. 7, line 6; U.S. Patent No. 4,912,038 to Schilling at Col. 3, line 26 to Col. 14, line 12; and U.S. Patent No. 4,879,224 to Wallner at Col. 6, line 8 to Col. 8, line 59.

15 The DNA encoding keratinase may be made according to any of the know techniques. For example, the DNA may be constructed using the MUTA-GENE™ phagemid in vitro mutagenesis kit by BIO-RAD. The kit is based on the method described by Kunkel in U.S. 20 Patent No. 4,873,192. (See also T. Kunkel, *Proc. Natl Acad. Sci. USA* **82**:488 (1985); T. Kunkel et al., *Methods in Enzymol.* **154**:367 (1987)). U.S. Patent No. 4,873,192 provides a very strong selected against the non-mutagenized strand of a double-stranded DNA. When DNA 25 is synthesized in a *dut-ung*-double mutant bacterium, the nascent DNA carries a number of uracils in thymine positions as a result of the *dut* mutation, which inactivates the enzyme dUTPase and results in high intracellular levels of dUTP. The *ung* mutation 30 inactivates uracil N-glycosylase, which allows the incorporated uracil to remain in the DNA. This uracil-containing strand is then used as the template for the *in vitro* synthesis of a complementary strand primed by an oligonucleotide containing the desired mutation. 35 When the resulting double-stranded DNA is transformed into a cell with a proficient uracil N-glycosylase, the

uracil-containing strand is inactivated with high efficiency, leaving the non-uracil-containing survivor to replicate (See generally, BIO-RAD catalog number 170-3576 instruction manual).

5 The keratinase gene encompassing the DNA encoding keratinase as well as regulatory elements may be constructed by amplification of a selected, or target, nucleic acid sequence. Amplification may be carried out by any suitable means. See generally, D.
10 Kwoh and T. Kwoh, *Am. Biotechnol. Lab.* 8:14 (1990). Examples of suitable amplification techniques include, but are not limited to polymerase chain reaction, ligase chain reaction, strand displacement
15 amplification (see generally, G. Walker et al., *Proc. Natl. Acad. Sci. USA* 89:392 (1992); G. Walker et al., *Nucleic Acids Res.* 20:1691 (1992)), transcription-based amplification (see, D. Kwoh et al., *Proc. Natl. Acad Sci. USA* 86:1173 (1989)), self-sustained sequence
20 replication (or "3SR") (see, J. Guatelli et al., *Proc. Natl. Acad. Sci. USA* 87:1874 (1990)), the Q β replicase system (see, P. Lizardi et al., *Biotechnology* 6:1197 (1988)), nucleic acid sequence-based amplification (or "NASBA") (see, R. Lewis, *Genetic Engineering News* 12
25 9:1 (1992)), the repair chain reaction (or "RCR") (see, R. Lewis, *supra*), and boomerang DNA amplification (or "BDA") (see R. Lewis, *supra*). Polymerase chain reaction is currently preferred.

DNA amplification techniques such as the foregoing can involve the use of a probe, a pair of
30 probes, or two pairs of probes which specifically bind to DNA encoding the desired target protein.

Polymerase chain reaction (PCR) may be carried out in accordance with known techniques. See, e.g., U.S. Patents Nos. 4,683,195; 4,683,202;
35 4,800,159; and 4,965,188. In general, PCR involves, first, treating a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) with one

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oligonucleotide primer for each strand of the specific sequence to be detected under hybridizing conditions so that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith so that the extension product synthesized from each primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and then treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present. These steps are cyclically repeated until the desired degree of amplification is obtained. Detection of the amplified sequence may be carried out by adding to the reaction product an oligonucleotide probe capable of hybridizing to the reaction product (e.g., an oligonucleotide probe of the present invention), the probe carrying a detectable label, and then detecting the label in accordance with known techniques, or by direct visualization on a gel.

Ligase chain reaction (LCR) is also carried out in accordance with known techniques. See, e.g., R. Weiss, *Science* **254**:1292 (1991). In general, the reaction is carried out with two pairs of oligonucleotide probes: one pair binds to one strand of the sequence to be detected; the other pair binds to the other strand of the sequence to be detected. Each pair together completely overlaps the strand to which it corresponds. The reaction is carried out by, first, denaturing (e.g., separating) the strands of the sequence to be detected, then reacting the strands with the two pairs of oligonucleotide probes in the presence of a heat stable ligase so that each pair of oligonucleotide probes is ligated together, then separating the reaction product, and then cyclically

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repeating the process until the sequence has been amplified to the desired degree. Detection may then be carried out in like manner as described above with respect to PCR.

5 A vector is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding a proteinase or keratinase as given herein and/or to express DNA which encodes a proteinase or keratinase as given herein. An expression vector is a replicable DNA
10 construct in which a DNA sequence encoding a proteinase or keratinase is operably linked to suitable control sequences capable of effecting the expression of the proteinase or keratinase in a suitable host. The need for such control sequences will vary depending upon the
15 host selected. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and
20 translation.

Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to
25 facilitate recognition of transformants.

Vectors comprise plasmids, viruses (e.g., adenovirus, cytomegalovirus), phage, and integratable DNA fragments (i.e., fragments integratable into the host genome by recombination). The vector replicates
30 and functions independently of the host genome, or may, in some instances, integrate into the genome itself. Expression vectors should contain a promoter and RNA polymerase binding sites which are operably linked to the gene to be expressed and are operable in the host
35 organism.

DNA regions are operably linked or operably associated when they are functionally related to each

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other. For example, a promoter is operably linked to or operably associated with a coding sequence if it controls the transcription of the sequence; or a ribosome binding site is operably linked to a coding
5 sequence if it is positioned so as to permit translation.

Transformed host cells are cells which have been transformed or transfected with vectors containing a DNA sequence as disclosed herein constructed using
10 recombinant DNA techniques. Transformed host cells ordinarily express the proteinase or keratinase, but host cells transformed for purposes of cloning or amplifying the proteinase or keratinase DNA do not need to express the proteinase or keratinase. Suitable host
15 cells can include host cells known to those skilled in the art, such as for example prokaryote host cells including *Bacillus subtilis*.

In the methods and systems of the present invention, *Bacillus subtilis* host cells are preferred.
20 *Bacillus subtilis* is capable of secreting enzymes extracellularly. (See generally, Priest, *Bacterial*. Rev. 41:711 (1977) and Doi et al., *Trends Biotechnol*. Sept. 232 (1986). This feature allows this bacterium to serve as a host cell for expression and secretion of
25 foreign proteins in the medium, which can be conveniently rendered to downstream processing and utilization. The *Bacillus subtilis* system has not been widely utilized, because either the inserted gene is poorly regulated in general, or foreign proteins are
30 likely to be hydrolyzed by high levels of proteases produced by *Bacillus subtilis*. *Bacillus subtilis* has six extracellular proteases, neutral protease A, subtilisin (or "alkaline protease"), extracellular protease, metalloprotease, bacillopeptidase F, and
35 neutral protease B. To overcome these problems, protease-deficient strains of *Bacillus subtilis* have been developed. (See generally, Doi et al., *Trends*

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Biotechnol. 4:232 (1986) and Wu et al. *J. Bacteriol.* 173:4952 (1991)). *Bacillus subtilis* deficient in only neutral protease, DB101, has been developed. A *Bacillus subtilis* strain deficient in two extracellular protease, namely neutral protease and alkaline protease, and known as DB104 has been developed. A *Bacillus subtilis* strain deficient in five proteases, known as GP263, has been developed and has eliminated much of the total extracellular protease activity. A *Bacillus subtilis* strain deficient in all six extracellular proteases, WB600, has also been constructed. Currently, DB104, or *Bacillus subtilis* deficient in two extracellular proteases, is the preferred strain for the host cells employed in the present invention.

Vectors for use in *Bacillus subtilis* host cells have been constructed. (See generally, Steinmetz et al., *Mol. Gen. Genet.* 200:220 (1985), Crutz et al., *J. Bacteriol.* 172:1043 (1990), and Wu et al., (1991) supra.) Preferably, *Bacillus subtilis* is transformed using vectors generated from pUB18 or pUB18-P43 plasmids. A promoter commonly used in these recombinant expression vectors include the strong vegetative promoter P43. The promoter is operably associated to the DNA encoding the keratinase, i.e., they are positioned so as to promote transcription of keratinase messenger RNA from the DNA.

The hyperexpression of keratinase has been observed using the *Bacillus subtilis* system where the *kerA* pre/pro processing and secretion region is inserted upstream of the DNA encoding keratinase. Hence, this is the preferred embodiment of the instant invention.

C. Production of Keratinase Enzyme

As noted above, keratinase enzyme can be made by culturing a host cell as described above under conditions that permit expression of the encoded

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keratinase, and collecting the expressed keratinase. The host cell may be cultured under conditions in which the cell grows, and then cultured under conditions which cause the expression of the encoded keratinase, or the cells may be caused to grow and express the encoded keratinase at the same time. The keratinase may be fused to an appropriate secretory leader sequence and secreted into the culture media and collected from the media, or the keratinase may be expressed intracellularly, the cells then lysed, and the keratinase collected from the cell lysate. Preferably, the enzyme is produced into the culture medium and collected therefrom. In general, any suitable techniques for culturing and expressing a transgenic protein may be used, as will be appreciated by those skilled in the art.

For example, the transformed *Bacillus subtilis* host cells may be cultured in Luria-Bertoni or feather medium, into which the expressed keratinase enzyme is secreted and from which the keratinase may be collected. The *Bacillus subtilis* host cells are typically cultured at temperatures ranging from 30 to 45°C. The expressed enzyme may be collected from the medium according to techniques widely known in the art. For example, the enzyme can be concentrated by ultrafiltration or ammonium sulfate precipitation, and purified by various chromatographic methods, as described in Lin et al., *Applied Environmental Microbiology* 58:3271 (1992).

According to one preferred embodiment of the present invention, keratinase is produced by (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith; and (b) collecting keratinase enzyme from said cell culture. According to one preferred embodiment, the vector DNA further

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comprises DNA encoding a *kerA* processing and secretion region. More preferably, the vector DNA further comprises a promoter, such as a P43 promoter, located upstream of the DNA encoding a *kerA* processing and secretion region. According to one preferred embodiment, the promoter is positioned in the same orientation as the DNA encoding the *Bacillus licheniformis* PWD-1 keratinase enzyme.

10 **D. Recombinant DNA and System for Expression of a Heterologous DNA**

The present invention also provides a recombinant DNA and host cell for expressing a heterologous DNA encoding an enzyme or protein. Typically the heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a proteinase. Preferably, the heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase. Examples of suitable heterologous DNA encoding enzymes for use in the present invention include but are not limited to proteases, amylase, lipase, hexose isomerase, β -gluconase, and phytase.

According to the present invention, the recombinant DNA comprises vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and the heterologous DNA encoding an enzyme or protein. The vector DNA typically comprises a promoter. Any suitable promoter capable of regulating the expression of the heterologous DNA in the selected host cell may be employed. Preferably, the promoter is a P43 promoter. In the preferred embodiment of the recombinant DNA of the present invention, the promoter is located upstream of the DNA encoding the *kerA* pre/pro processing and secretion region and is in the same orientation as the heterologous DNA encoding the enzyme or protein.

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The recombinant DNA may be transfected into a host cell to provide a host cell capable of expressing the heterologous DNA. Suitable host cells include those host cells discussed hereinabove in connection with the expression and secretion of keratinase. The preferred host cell is *Bacillus subtilis*, and particularly the *Bacillus subtilis* strain which is deficient in both neutral and alkaline cellular proteases. The recombinant DNA of the present invention and the host cell provide a *Bacillus* system for the expression and secretion of an enzyme or protein encoded by a heterologous DNA.

E. Methods of Expressing Heterologous DNA

The present invention also provides methods of expressing a heterologous DNA encoding an enzyme or protein. The methods of the present invention include (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme or protein, and (b) collecting enzyme or protein from the *Bacillus subtilis* host cell culture or cell culture medium. The recombinant DNA and host cell of the present invention are described in further detail hereinabove.

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, "g" means grams, "μg" means micrograms, "l" means liters, "ml" means milliliters, "g/l" means grams per liter, "μg/ml" means micrograms per milliliter, "°C" means degrees Centigrade, "Km" means kanamycin.

Bacillus licheniformis PWD-1 has the accession number ATCC 53757. *Bacillus licheniformis* PWD-1 was grown on either 1) feather medium consisting of 0.5 g/l of sodium chloride, 0.1 g/l magnesium

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chloride hexahydrate, 0.06 g/l calcium chloride, 0.7 g/l KH_2PO_4 , 1.4 g/l K_2HPO_4 , 1.0 g/l tryptone, and 10 g/l chopped feathers at pH 7.0; or 2) Luria-Bertani ("LB") medium at 50°C. *Bacillus subtilis* DB104 is grown according to Kawamura and Doi, *J. Bacteriol.* **160**:442 (1984) and is deficient in both alkaline and neutral extracellular proteases. Specifically, *B. subtilis* DB104 was grown at 37°C on LB medium. *B. subtilis* DB104 carrying plasmid pUB18 or its derivatives, Km was added to the medium at a final concentration of 20 $\mu\text{g}/\text{ml}$. *Escherichia coli* INV α F' and PCR cloning vector, pCRII, were purchased from Invitrogen Corporation, San Diego, California. *E. coli* INV α F' was grown at 37°C on LB medium supplemented with 50 $\mu\text{g}/\text{ml}$ ampicillin. TBAB plates containing 20 μg Km/ml were obtained from Difco Laboratories, Detroit, Michigan and used for routine transformation. A skim milk-feather powder plate (containing 5% skim milk, 0.5% feather powder, 1% agar, and 20 μg Km/ml) were used to screen colonies producing keratinase. Transformed *B. subtilis* strains were grown at 37°C on LB medium or feather medium.

EXAMPLE 1

DNA Manipulations

Mini-preparation of plasmids of pUB18, pUB18-P43 and their derivatives are prepared by rapid alkaline sodium dodecyl sulfate method, according to the method of Rodriguez, Recombinant DNA Techniques, Addison-Wesley Publishing Co., (1983), the disclosure of which is incorporated herein by reference in its entirety. The 1.4 kb *kerA* fragment is cloned into polylinker site of plasmid pCRII and stored in *E. Coli* INV α F' as described previously by Lin et al., *Applied Environmental Microbiology* **61**:1469 (1995), the disclosure of which is incorporated herein by reference in its entirety. After *E. coli* INV α F' cells are grown on LB medium overnight, plasmid pCRII with *kerA* is

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extracted by several mini-preparations, pooled and excised for *kerA* by *XbaI* and *SpeI* digestion. The digestion mixture is applied on 1.2% agarose gel electrophoresis for separation. *kerA* band is cut out, and extracted from the gel by using an Elu-Quik DNA purification kit purchased from Schleicher & Schuell, Keene, New Hampshire. The extraction is carried out following the manufacturer's instruction. All restriction enzymes are the products of Promega Corporation, Madison, Wisconsin. The construction of plasmid pLB3 containing *kerA* is set forth in **Figure 1**. *Km^r* represents the kanamycin resistance gene. Arrows indicate the orientations of the genes.

EXAMPLE 2

Construction of Vectors

Plasmid pUB18-P43 is created by inserting a DNA fragment (~300 bp) containing vegetative promoter P43 as described in Wang, et al., *Journal of Biological Chemistry* 259:8619 (1984), adjacent to the polycloning site of pUB18. Both plasmids pUB18 and pUB18-P43 have the same polycloning site available for gene insertion. When the plasmids are digested by *HindIII* (5'-AAGCTT-3'), four-base overhangs (5'-AGCT-3') are generated on both ends. Partial fill in with nucleotides A and G generated two-nucleotides overhangs (5'-AG-3') at the ends of the linearized vectors. The 1.4-kb *kerA* fragment in pCRII flanking by *XbaI* (5'-TCTAGA-3') and *SpeI* (5'-ACTAGT-3') recognition site was excised by *XbaI-SpeI* digestion. The same single-strand overhangs (5'-CTAG-3') are generated at both ends. Again, partial fill in with nucleotides T and C created another two-nucleotide overhangs (5'-CT-3') at both ends of the insert. These two separate treatments produced complementary overhangs on the vectors and insert as illustrated in **Figure 1**. Vector and insert in a molar ratio of 1:2 are mixed and ligated according

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to the method of Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, the disclosure of which is incorporated herein by

5 reference.

The structures of plasmids pLB3, pLB29, and pLB36 are set forth in **Figure 2**.

EXAMPLE 3

Cloning and Screening

10 The linearized pUB18 and pUB18-P43 created by *Hind*III digestion were flanked by overhangs 5'-AGCT-3', which is not complementary with the overhangs on *kerA* fragment generated by *Xba*I-*Spe*I digestion. However, the fill-in treatments on vectors by AG and on insert
15 by CT generated complementary two-nucleotide overhangs between vectors and *kerA* fragment to facilitate the ligation. Fill in also prevented linearized vector from religation, which reduced background colonies dramatically during the transformants screening. Using
20 skim milk-feather powder plates proved to be an efficient means of selecting transformants capable of expressing *kerA*.

EXAMPLE 3

Preparation of *B. subtilis* Competent Cells

25 *B. subtilis* DB104 competent cells are prepared as described in Dubnau et al., *Journal of Molecular Biology* 56:209 (1971), the disclosure of which is incorporated herein by reference in its entirety. *B. Subtilis* cells grown overnight on TBAB
30 plates are inoculated with 2 ml of SP1 medium according to J Spizizen, *Proc. Natl. Acad. Sci. USA* 44:1072 (1958) and Dubnau et al., *Journal of Molecular Biology* 56:209 (1971). The SP1 medium is prepared with 0.2% $(\text{NH}_4)_2\text{SO}_4$, 1.4% K_2HPO_2 , 0.6% KH_2PO_4 , 0.1% sodium
35 citrate $\cdot 2\text{H}_2\text{O}$, 0.02% MgSO_4 , 0.02% casamino acids, 0.1%

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yeast extract, 0.005% tryptophan. One ml of pre-filtrated (0.2 μ membrane) 50% glucose solution per 100 ml of SP1 medium is added after the medium is autoclaved. Cells are grown at 37°C for 3.5 to 4 hours with rapid shaking at 300 rpm. A 0.5 ml culture of SP1 medium is then transferred to 4.5 ml SP2 medium (SP1 medium with additional 0.5 mM CaCl₂ and 2.5 mM MgCl₂), and grown for an additional 90 minutes. Thereafter, 50 μ l of EGTA solution (100 mM EGTA, pH 7.0) is added to the SP2 medium. The cells are ready for transformation after shaking for 10 minutes.

EXAMPLE 4

Transformation of *B. subtilis* DB104 and Screening for Colonies Harboring Plasmid

Ligated DNA in 50 μ l is added to 0.5 ml of freshly prepared *B. subtilis* DB104 competent cells. After shaking at 200 rpm at 37°C for 90 minutes, cells are plated on TBAB plates with 20 μ g Km/ml, and incubated at 37°C overnight. Colonies grown on TBAB plates are transferred to skim-milk-agar plates for further selection. The colonies having clear haloes are selected for plasmid isolation and analysis.

Transformation of *B. subtilis* using ligated pUB18-kerA and pUB18-P43-kerA DNA yielded hundreds of colonies on TBAB plates. Thirty six from each group are randomly selected and transferred onto skim milk-agar feather powder plates for a secondary selection. Seven colonies from pUB18-kerA transformant group and six colonies from pUB18-P43-kerA transformant group produced clear halos around colonies in 10 hour incubation at 37°C, while DB104/pUB18 and DB104/pUB18-P43 cells as controls did not show any sign of protein hydrolysis even after 48 hours. Those transformants cells are then grown in LB medium containing 20 μ g km/ml for 3 hours. Cells in 2 ml culture from each clone are used for plasmid isolation.

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EXAMPLE 5**Analysis of Plasmid Constructs**

All plasmids isolated from halo-forming colonies displayed a 1.4 kb increase in size. When the
5 plasmids were used as templates for PCR amplifications, 1.4 kb fragments were produced in the reactions priming by Primer I and Primer II. These results confirmed that the increase in size by 1.4 kb is due to the insertion of *kerA*.

10 Plasmids pLB3, pLB29, and pLB 36 represent all new vectors isolated from halo-forming colonies. In fact, pLB3 represents all plasmids isolated from pUB18-*kerA* group because all of them have the *kerA* in the same orientation. In the pUB-P43-*kerA* group, pLB29
15 and pLB36 represent two opposite orientations of *kerA*.

To determine the orientation of *kerA* in the plasmids, Primer III was combined with either Primer I or Primer II to perform PCR amplifications. When pLB3 and pLB36 as templates, and Primer I and Primer III are
20 used, PCR amplified a 1.5 kb fragment and a 1.8 kb fragment respectively. The increases in size were due to the amplification of an additional 52 bp from original pUB18 and ~350 bp from original pUB18-P43. The presence of PCR products also proved that *kerA* in
25 pLB3 and pLB36 have the same orientation, and that they have the same orientation as the kanamycin resistance gene (*Km^r*) on the vectors. PCR using pLB29 template and Primer I and Primer III did not produce any major DNA fragment. However, when Primer I is replaced by Primer
30 II, a 1.8 kb fragment is observed on the agarose gel. These results indicate that *kerA* in pLB29 is in the same orientation with P43 promoter, but opposite to *Km^r*.

EXAMPLE 6**Identification of *kerA* in Plasmids**

35 The newly constructed plasmids are digested by *XbaI*, followed by 1.2% agarose gel analysis.

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Plasmids with a 1.4 kb size increase are applied to PCR amplifications. Three PCR primers are synthesized: Primer I (5'-CTCCTGCCAAGCTGAAGC-3', 18 mers) (SEQ ID NO.:3) and Primer II (5'-GATCATGGAACGGATTC-3', 17 mers) (SEQ ID NO.:4), which are homologous to the upstream and downstream of *kerA*, respectively and Primer III (5'-GCCGTCTGTACGTTCTAAG-3', 20 mers) (SEQ ID NO.:5) which is derived from the upstream DNA sequence of the polycloning site on pUB18 and pUB18-P43. PCR amplifications with any two of the given primers are performed as described in Lin et al., *Applied Environmental Microbiology* 61:1469 (1995)., the disclosure of which is incorporated herein by reference in its entirety; except that the newly constructed plasmids are used as templates. Approximately 156 ng plasmid DNA is used as the template in each PCR reaction.

EXAMPLE 7

Expression of *kerA* in LB and Feather Media

Five strains, *B. subtilis* DB104/pUB18, FDB-3 (DB104/pLB3), FDB-29 (DB104/pLB29), FDB-36 (DB104/pLB36), and *B. licheniformis* PWD-1 grew rapidly in LB medium. At 36 hours, 40 μ l of supernatant from each medium is loaded into small wells on milk-agar plate, and incubated at 50°C overnight. Hydrolysis haloes are only observed around the wells in which supernatants from FDB-3, FDB-29, and FDB-36 are loaded. This result is confirmed by the azo-keratin hydrolysis assay, when a 0.2 ml sample of each medium is taken at every 4 hours and determined for its keratinolytic activity. Again media from all three strains showed strong activities against azokeratin, and FDB-29 gives the highest activity among all the three transformants. Both PWD-1 and DB104/pUB18 media showed no proteolytic activities.

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All five strains are also tested in feather media. PWD-1 and FDB-29 grew rapidly and reach their highest keratinase activity in approximately 72 hours. FDB-3, and FDB-36 did not display significant
5 keratinolytic activities until the third day, reaching their highest activities at least 24 hours later than FDB-29 did. FDB-29 still demonstrated the highest activity, which was 3 to 4 fold higher than that of PWD-1 grown on feather media at 50°C. PWD-1 showed
10 positive results only in feather media. DB104, the host strain, does not produce keratinase in either LB or feather media.

In feather media all these new strains, FDB-3, FDB-29, and FDB-36 yielded more keratinolytic
15 activity when kanamycin was not added in the medium. FDB-29 produced more keratinase in LB medium without this antibiotic. FDB-3 and FDB-36 however, demonstrated higher keratinolytic activity in LB medium when kanamycin was added.

20

EXAMPLE 8

Detection of Keratinase Activity

Two methods, hydrolysis of azokeratin and milk-agar plate assay, are used to detect keratinase activity according to the methods described in Lin et
25 al., *Applied Environmental Microbiology* 58:3271 (1992), the disclosure of which is incorporated herein by reference in its entirety. SDS gel electrophoresis is conducted as described in Laemmili, et al., *Nature* 227:680 (1970), the disclosure of which is incorporated
30 herein by reference in its entirety. Purified keratinase is used to generate anti-keratinase serum in rabbits by the standard method described in Harlow et al., Antibodies, A Laboratory Manual (1988) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
35 This anti-serum which precipitates with keratinase is used to detect the enzyme in agar gel. DNA restriction

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and agarose gel electrophoresis are performed as described by Sambrook et al., Molecular Cloning, A Laboratory Manual 2nd ed. (1988).

EXAMPLE 9

5 Confirmation of Expression of *kerA*

Active keratinase was produced by FDB-3, FDB-29, and FDB-36 in LB and feather media. This has been confirmed by milk-agar plate (containing 4% evaporated skim-milk, 1.5% agar, and 0.02% sodium azide) assay.

10 **Figure 3** illustrates the detection of proteolytic activity by formation of hydrolysis haloes on milk-agar plates. Plate A contains cell-free culture supernatants from feather medium. *Bacillus* *licheniformis* PWD-1 and FDB-29 samples were taken at 72
15 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium. PWD-1 was grown at 50°C and all others were grown at 37°C.

Confirmation of the production of active
20 keratinase by FDB-3, FDB-29 and FDB-36 in LB and feather media was also obtained by azokeratine hydrolysis as illustrated by **Figure 4**. The assay was carried out in 500 ml flask with 150 ml medium. Seed cultures of FDB-3, FDB-29, FDB-36 and DB104/pUB18 were
25 grown in 10 ml LB medium with 20 µg Km/ml for 4 hours, and 1 ml of each was inoculated to 150-ml flask feather and LB media. Seed culture of PWD-1 (10 ml) were grown on LB and feather media for overnight firstly, and 1 ml of each was inoculated to LB and
30 feather media, respectively. No kanamycin was added into feather medium or PWD-1 growth media. Keratinolytic activity was measured according to the methods described in Lin et al., *Appl. Environ. Microbiol.* 58:3271 (1992).

35 Confirmation of the production of active keratinase by FDB-3, FDB-29 and FDB-36 in LB and

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feather media was also obtained by immuno-precipitation assay as illustrated by **Figure 5**. The rabbit anti-keratinase serum was loaded into the holes in the center of each plate. Plate A contains cell-free culture supernatants from feather medium. *Bacillus licheniformis* PWD-1 and FDB-29 samples were taken at 72 hours, FDB-3, FDB-36, and DB104/pUB18 samples were taken at 96 hours. Plate B contains 36-hour cell-free culture supernatants from LB medium.

10 The double immuno-diffusion results indicate that FDB-3, FDB-29, and FDB-36 produced keratinase in both LB and feather media. PWD-1 in LB media and DB104 in both feather and LB media have negative responses in this immuno-precipitation assay.

15 Confirmation of keratinase activity was also achieved using SDS-polyacrylamide gel electrophoresis. The 33 kDa keratinase bands appeared on SDS-polyacrylamide gel when the media of FDB-3, FDB-29, and FDB-36 are used.

20

EXAMPLE 10

Effects of Promoter Orientation

As discussed in **Example 6**, PCR amplification analysis illustrated that P43 was installed upstream of *kerA* in pLB29 and in the same orientation as *kerA*.

25 pLB36 has the P43 promoter in the opposite orientation from *kerA* and pLB3 does not contain the P43 promoter. The results of the keratinolytic activity of FDB-29, FDB-36, and FDB-3 cells demonstrate that the P43 promoter greatly enhanced the expression of *kerA*.

30 Rapid cell growth of FDB-29 cells, associated with keratinolytic activity increase, was observed in feather medium. In contrast, FDB-3 and FDB-36 in feather medium show a long adaptive period, and produce most of their enzymes after 4 days of culture.

35 Although the inventors do not wish to be bound by any particular theory, it appears that FDB-3 and FDB-36

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underwent an induction process, which resulted in the eventual expression of *kerA*.

EXAMPLE 11

Effects of Orientation of Kanamycin Resistance Gene

5 The kanamycin resistance gene (Km^r) carried by plasmid expresses in response to kanamycin in the medium, and has an influence on the expression of *kerA*. In the presence of kanamycin, in both LB and feather media, FDB-29 produced slightly low activities, as
10 reported in **Figure 6B**. The decrease in *kerA* expression may be due to the generation of antisense RNA resulting from the readthrough of the kanamycin resistance gene. For FDB-3 and FDB-36, the increase in expression of
15 *kerA* may also be caused by the same readthrough of the kanamycin resistance gene, since *kerA* and the kanamycin resistance gene in these two vectors are in the same orientation. The results for FDB-3 and FDB-36 are reported in **Figures 6A** and **6C** respectively. The same increases were not found when FDB-3 and FDB-36 were
20 grown in the feather medium. It is possible that the induction of *kerA* expression is crucial when they are grown on feathers. No keratinolytic activity was produced by *Bacillus licheniformis* PWD-1 in LB medium.

EXAMPLE 12

25 **Secretion of Keratinase in Protease-Deficient *Bacillus subtilis***

 The DB104 host cells employed in the foregoing experiments are deficient in two major extracellular proteases, neutral and alkaline
30 proteases. The results of keratinolytic activity indicate that DB104 is able to express *kerA* originating from *Bacillus licheniformis* strain and secrete active keratinase into the medium at a high level. Because *kerA* pre/pro processing and secretion region exist
35 upstream of the keratinase structure gene, premature

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keratinase in the cell must have been processed to active enzyme. These results demonstrate that the *kerA* pre/pro processing and secretion region is recognized and processed in DB104 even though it is deficient in
5 two major cellular proteases.

A similar *Bacillus subtilis*, WB600, which is deficient in six cellular proteases was also tested for expression of *kerA* in pLB29. Low enzyme activity was produced in LB medium. These results suggest that for
10 effective production of foreign protein, the host cell with high levels of extracellular proteases is harmful, but a low level of proteolytic process may be necessary for activating enzymes by limited proteolysis.

The foregoing is illustrative of the present
15 invention and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Shih, Jason C.H.
Lin, Xiang
Wong, Sui-Lam
- (ii) TITLE OF INVENTION: Method For Expressing and Secreting
Keratinase
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Kenneth D. Sibley
 - (B) STREET: P.O. Drawer 31107
 - (C) CITY: Raleigh
 - (D) STATE: North Carolina
 - (E) COUNTRY: USA
 - (F) ZIP: 27622
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0. Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Sibley, Kenneth D.
 - (B) REGISTRATION NUMBER: 31.665
 - (C) REFERENCE/DOCKET NUMBER: 5051-304
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (919) 420-2200
 - (B) TELEFAX: (919) 881-3175

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1457 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:

-29-

(A) NAME/KEY: CDS
 (B) LOCATION: 215..1351

(ix) FEATURE:
 (A) NAME/KEY: mat_peptide
 (B) LOCATION: 530..1351

(ix) FEATURE:
 (A) NAME/KEY: misc RNA
 (B) LOCATION: 215..301
 (D) OTHER INFORMATION: /note= "pre-region of keratinase"

(ix) FEATURE:
 (A) NAME/KEY: misc RNA
 (B) LOCATION: 302..529
 (D) OTHER INFORMATION: /note= "pro-region of keratinase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATTTTTTTTC TATAATAAAT TAACAGAATA ATTGGAATAG ATTATATTAT CCTTCTATTT	180
AAATTATTCT GAATAAAGAG GAGGAGAGTG AGTA ATG ATG AGG AAA AAG AGT	232
Met Met Arg Lys Lys Ser	
-105 -100	
TTT TGG CTT GGG ATG CTG ACG GCC TTC ATG CTC GTG TTC ACG ATG GCA	280
Phe Trp Leu Gly Met Leu Thr Ala Phe Met Leu Val Phe Thr Met Ala	
-95 -90 -85	
TTC AGC GAT TCC GCT TCT GCT GCT CAA CCG GCG AAA AAT GTT GAA AAG	328
Phe Ser Asp Ser Ala Ser Ala Ala Gln Pro Ala Lys Asn Val Glu Lys	
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GAT TAT ATT GTC GGA TTT AAG TCA GGA GTG AAA ACC GCA TCT GTC AAA	376
Asp Tyr Ile Val Gly Phe Lys Ser Gly Val Lys Thr Ala Ser Val Lys	
-65 -60 -55	
AAG GAC ATC ATC AAA GAG AGC GGC GGA AAA GTG GAC AAG CAG TTT AGA	424
Lys Asp Ile Ile Lys Glu Ser Gly Gly Lys Val Asp Lys Gln Phe Arg	
-50 -45 -40	
ATC ATC AAC GCG GCA AAA GCG AAG CTA GAC AAA GAA GCG CTT AAG GAA	472
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GTC AAA AAT GAT CCG GAT GTC GCT TAT GTG GAA GAG GAT CAT GTG GCC	520
Val Lys Asn Asp Pro Asp Val Ala Tyr Val Glu Glu Asp His Val Ala	
-15 -10 -5	
CAT GCC TTG GCG CAA ACC GTT CCT TAC GGC ATT CCT CTC ATT AAA GCG	568

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GTC	CTG	GAT	ACA	GGA	ATC	CAA	GCT	TCT	CAT	CCG	GAC	TTG	AAC	GTA	GTC	664	
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GTA	CTG	AAT	TCA	AGC	GGA	AGC	GGA	TCA	TAC	AGC	GGC	ATT	GTA	AGC	GGA	856	
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Ile	Glu	Trp	Ala	Thr	Thr	Asn	Gly	Met	Asp	Val	Ile	Asn	Met	Ser	Leu		
	110				115					120					125		
GGG	GGA	GCA	TCA	GGC	TCG	ACA	GCG	ATG	AAA	CAG	GCA	GTC	GAC	AAT	GCA	952	
Gly	Gly	Ala	Ser	Gly	Ser	Thr	Ala	Met	Lys	Gln	Ala	Val	Asp	Asn	Ala		
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Tyr	Ala	Arg	Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Ser	Gly	Ser		
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Ser	Gly	Asn	Thr	Asn	Thr	Ile	Gly	Tyr	Pro	Ala	Lys	Tyr	Asp	Ser	Val		
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ATC	GCT	GTT	GGT	GCG	GTA	GAC	TCT	AAC	AGC	AAC	AGA	GCT	TCA	TTT	TCC	1096	
Ile	Ala	Val	Gly	Ala	Val	Asp	Ser	Asn	Ser	Asn	Arg	Ala	Ser	Phe	Ser		
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AGT	GTG	GGA	GCA	GAG	CTT	GAA	GTC	ATG	GCT	CCT	GGC	GCA	GGC	GTA	TAC	1144	
Ser	Val	Gly	Ala	Glu	Leu	Glu	Val	Met	Ala	Pro	Gly	Ala	Gly	Val	Tyr		
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AGC	ACT	TAC	CCA	ACG	AAC	ACT	TAT	GCA	ACA	TTG	AAC	GGA	ACG	TCA	ATG	1192	
Ser	Thr	Tyr	Pro	Thr	Asn	Thr	Tyr	Ala	Thr	Leu	Asn	Gly	Thr	Ser	Met		
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225 230 235	
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Thr Tyr Leu Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val	
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GAA GCT GCC GCT CAA TAACATATTC TAACAAATAG CATATAGAAA AAGCTAGTGT	1391
Glu Ala Ala Ala Gln	
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ATGATC	1457

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 379 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Met Arg Lys Lys Ser Phe Trp Leu Gly Met Leu Thr Ala Phe Met	-105	-100	-95	-90
Leu Val Phe Thr Met Ala Phe Ser Asp Ser Ala Ser Ala Ala Gln Pro	-85	-80	-75	
Ala Lys Asn Val Glu Lys Asp Tyr Ile Val Gly Phe Lys Ser Gly Val	-70	-65	-60	
Lys Thr Ala Ser Val Lys Lys Asp Ile Ile Lys Glu Ser Gly Gly Lys	-55	-50	-45	
Val Asp Lys Gln Phe Arg Ile Ile Asn Ala Ala Lys Ala Lys Leu Asp	-40	-35	-30	
Lys Glu Ala Leu Lys Glu Val Lys Asn Asp Pro Asp Val Ala Tyr Val	-25	-20	-15	-10
Glu Glu Asp His Val Ala His Ala Leu Ala Gln Thr Val Pro Tyr Gly	-5	1	5	
Ile Pro Leu Ile Lys Ala Asp Lys Val Gln Ala Gln Gly Phe Lys Gly	10	15	20	

-32-

Ala Asn Val Lys Val Ala Val Leu Asp Thr Gly Ile Gln Ala Ser His
 25 30 35
 Pro Asp Leu Asn Val Val Gly Gly Ala Ser Phe Val Ala Gly Glu Ala
 40 45 50 55
 Tyr Asn Thr Asp Gly Asn Gly His Gly Thr His Val Ala Gly Thr Val
 60 65 70
 Ala Ala Leu Asp Asn Thr Thr Gly Val Leu Gly Val Ala Pro Ser Val
 75 80 85
 Ser Leu Tyr Ala Val Lys Val Leu Asn Ser Ser Gly Ser Gly Ser Tyr
 90 95 100
 Ser Gly Ile Val Ser Gly Ile Glu Trp Ala Thr Thr Asn Gly Met Asp
 105 110 115
 Val Ile Asn Met Ser Leu Gly Gly Ala Ser Gly Ser Thr Ala Met Lys
 120 125 130 135
 Gln Ala Val Asp Asn Ala Tyr Ala Arg Gly Val Val Val Val Ala Ala
 140 145 150
 Ala Gly Asn Ser Gly Ser Ser Gly Asn Thr Asn Thr Ile Gly Tyr Pro
 155 160 165
 Ala Lys Tyr Asp Ser Val Ile Ala Val Gly Ala Val Asp Ser Asn Ser
 170 175 180
 Asn Arg Ala Ser Phe Ser Ser Val Gly Ala Glu Leu Glu Val Met Ala
 185 190 195
 Pro Gly Ala Gly Val Tyr Ser Thr Tyr Pro Thr Asn Thr Tyr Ala Thr
 200 205 210 215
 Leu Asn Gly Thr Ser Met Val Ser Pro His Val Ala Gly Ala Ala Ala
 220 225 230
 Leu Ile Leu Ser Lys His Pro Asn Leu Ser Ala Ser Gln Val Arg Asn
 235 240 245
 Arg Leu Ser Ser Thr Ala Thr Tyr Leu Gly Ser Ser Phe Tyr Tyr Gly
 250 255 260
 Lys Gly Leu Ile Asn Val Glu Ala Ala Ala Gln
 265 270

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

-33-

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCCTGCCAA GCTGAAGC

18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCATGGAA CGGATTC

17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCGTCTGTA CGTTCCTAAG

20

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That Which Is Claimed Is:

1. A *Bacillus subtilis* host cell containing a recombinant DNA molecule, wherein said recombinant DNA molecule comprises vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme
5 operatively associated therewith, and capable of expressing and secreting keratinase.
2. The *Bacillus subtilis* host cell according to Claim 1, wherein said vector DNA of said recombinant DNA molecule further comprises a *kerA*
10 pre/pro processing and secretion region.
3. The *Bacillus subtilis* host cell according to Claim 1, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.
4. The *Bacillus subtilis* host cell
15 according to Claim 3, wherein said promoter is positioned upstream from said DNA encoding said *kerA* pre/pro processing and secretion region, and is operatively associated therewith.
5. The *Bacillus subtilis* host cell
20 according to Claim 4, wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.
6. The *Bacillus subtilis* host cell according to Claim 3, wherein said promoter is a P43
25 promoter.
7. The *Bacillus subtilis* host cell according to Claim 1, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

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8. The *Bacillus subtilis* host cell according to Claim 1, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

5 9. A method of producing keratinase enzyme comprising:

- (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1
10 keratinase enzyme operatively associated therewith; and
(b) collecting keratinase enzyme from said cell culture.

10. The method according to Claim 9, wherein said vector DNA of said recombinant DNA molecule
15 further comprises a *kerA* pre/pro processing and secretion region.

11. The method according to Claim 9, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

20 12. The method according to Claim 11, wherein said promoter is positioned upstream from a *kerA* pre/pro processing and secretion region and is operatively associated therewith.

13. The method according to Claim 11,
25 wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.

14. The method according to Claim 11, wherein said promoter is a P43 promoter.

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15. The method according to Claim 9, wherein said step of collecting keratinase enzyme comprises separating said enzyme from said cell culture by a method selected from the group consisting of
5 ultrafiltration and ammonium sulfate precipitation.

16. An expression and secretion system for keratinase enzyme comprising:

- (a) a *Bacillus subtilis* host cell, and
- (b) a recombinant DNA molecule comprising
10 vector DNA, DNA encoding a *ker A* pre/pro processing and secretion region, and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith.

17. The expression and secretion system
15 according to Claim 16, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

18. The expression and secretion system according to Claim 17, wherein said promoter is located upstream from said *kerA* pre/pro processing and
20 secretion region and operatively associated therewith.

19. The expression and secretion system according to Claim 17, wherein said promoter is in the same orientation as said DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme.

20. The expression and secretion system
25 according to Claim 17, wherein said promoter is a P43 promoter.

21. The expression and secretion system according to Claim 16, wherein said *Bacillus subtilis*
30 host cell is deficient in at least one protease.

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22. The expression and secretion system according to Claim 16, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

5 23. A recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode
10 *Bacillus licheniformis* PWD-1 keratinase enzyme.

24. The recombinant DNA molecule according to Claim 23, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a proteinase.

15 25. The recombinant DNA molecule according to Claim 23, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA encoding a keratinase.

20 26. The recombinant DNA molecule according to Claim 23, wherein said vector DNA further comprises a promoter.

27. The recombinant DNA molecule according to Claim 26, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing
25 and secretion region, and is operatively associated therewith.

28. The recombinant DNA molecule according to Claim 26, wherein said promoter is in the same orientation as said heterologous DNA encoding said
30 enzyme.

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29. The recombinant DNA molecule according to Claim 26, wherein said promoter is a P43 promoter.

30. A *Bacillus subtilis* host cell containing a recombinant DNA molecule, wherein said recombinant
5 DNA molecule comprises vector DNA, DNA encoding a *kerA* pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme; wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1
10 keratinase enzyme, and wherein said host cell is capable of expressing and secreting an enzyme encoded by said heterologous DNA.

31. The *Bacillus subtilis* host cell according to Claim 30, wherein said heterologous DNA
15 encoding an enzyme comprises a heterologous DNA encoding a proteinase.

32. The *Bacillus subtilis* host cell according to Claim 30, wherein said heterologous DNA encoding an enzyme comprises a heterologous DNA
20 encoding a keratinase.

33. The *Bacillus subtilis* host cell according to Claim 30, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

34. The *Bacillus subtilis* host cell
25 according to Claim 33, wherein said promoter is positioned upstream from said DNA encoding said *kerA* processing and secretion region, and is operatively associated therewith.

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35. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is in the same orientation as said heterologous DNA encoding said enzyme.

5 36. The *Bacillus subtilis* host cell according to Claim 33, wherein said promoter is a P43 promoter.

37. The *Bacillus subtilis* host cell according to Claim 30, wherein said *Bacillus subtilis*
10 host cell is deficient in at least one protease.

38. The *Bacillus subtilis* host cell according to Claim 30, wherein said *Bacillus subtilis* host cell is deficient in both neutral and alkaline proteases.

15 39. A method of producing an enzyme comprising the steps of:

(a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA, DNA encoding a *kerA* pre/pro processing and
20 secretion region, and a heterologous DNA encoding an enzyme, wherein said heterologous DNA encoding said enzyme is a heterologous DNA which does not encode *Bacillus licheniformis* PWD-1 keratinase enzyme; and

(b) collecting enzyme from said *Bacillus*
25 *subtilis* host cell culture.

40. The method according to Claim 39, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a proteinase.

41. The method according to Claim 39,
30 wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a keratinase.

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42. The method according to Claim 39,
wherein said vector DNA further comprises a promoter.

43. The method according to Claim 42,
wherein said promoter is positioned upstream from said
5 DNA encoding said *kerA* processing and secretion region,
and is operatively associated therewith.

44. The method according to Claim 42,
wherein said promoter is in the same orientation as
said heterologous DNA encoding said enzyme.

10 45. The method according to Claim 42,
wherein said promoter is a P43 promoter.

46. The method according to Claim 39,
wherein said step of collecting said enzyme comprises
separating said enzyme from said cell culture by a
15 method selected from the group consisting of
ultrafiltration and ammonium sulfate precipitation..

47. An expression and secretion system for
an enzyme comprising:
(a) a *Bacillus subtilis* host cell, and
20 (b) a recombinant DNA molecule comprising
vector DNA, DNA encoding a *kerA* pre/pro processing and
secretion region, and a heterologous DNA encoding an
enzyme, wherein said heterologous DNA encoding said
enzyme is a heterologous DNA which does not encode
25 *Bacillus licheniformis* PWD-1 keratinase enzyme.

48. The expression and secretion system
according to Claim 47, wherein said heterologous DNA
encoding said enzyme comprises a heterologous DNA
encoding a proteinase.

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49. The expression and secretion system according to Claim 47, wherein said heterologous DNA encoding said enzyme comprises a heterologous DNA encoding a keratinase.

5 50. The expression and secretion system according to Claim 47, wherein said vector DNA of said recombinant DNA molecule further comprises a promoter.

51. The expression and secretion system according to Claim 50, wherein said promoter is located
10 upstream from said *kerA* pre/pro processing and secretion region, and is operatively associated therewith.

52. The expression and secretion system according to Claim 50, wherein said promoter is in the
15 same orientation as said heterologous DNA encoding said enzyme.

53. The expression and secretion system according to Claim 50, wherein said promoter is a P43 promoter.

20 54. The expression and secretion system according to Claim 47, wherein said *Bacillus subtilis* host cell is deficient in at least one protease.

55. The expression and secretion system according to Claim 47, wherein said *Bacillus subtilis*
25 host cell is deficient in both neutral and alkaline proteases.

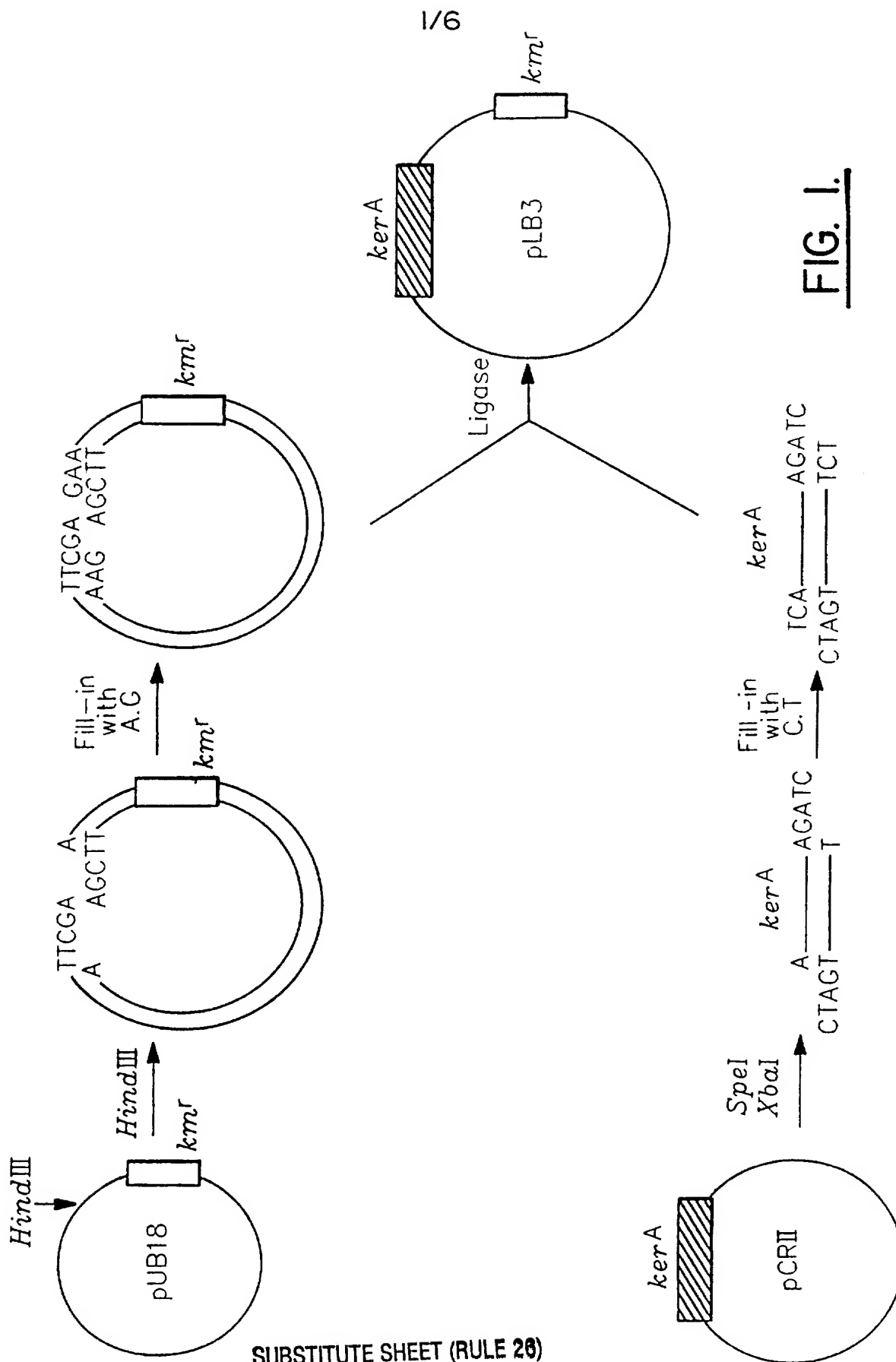
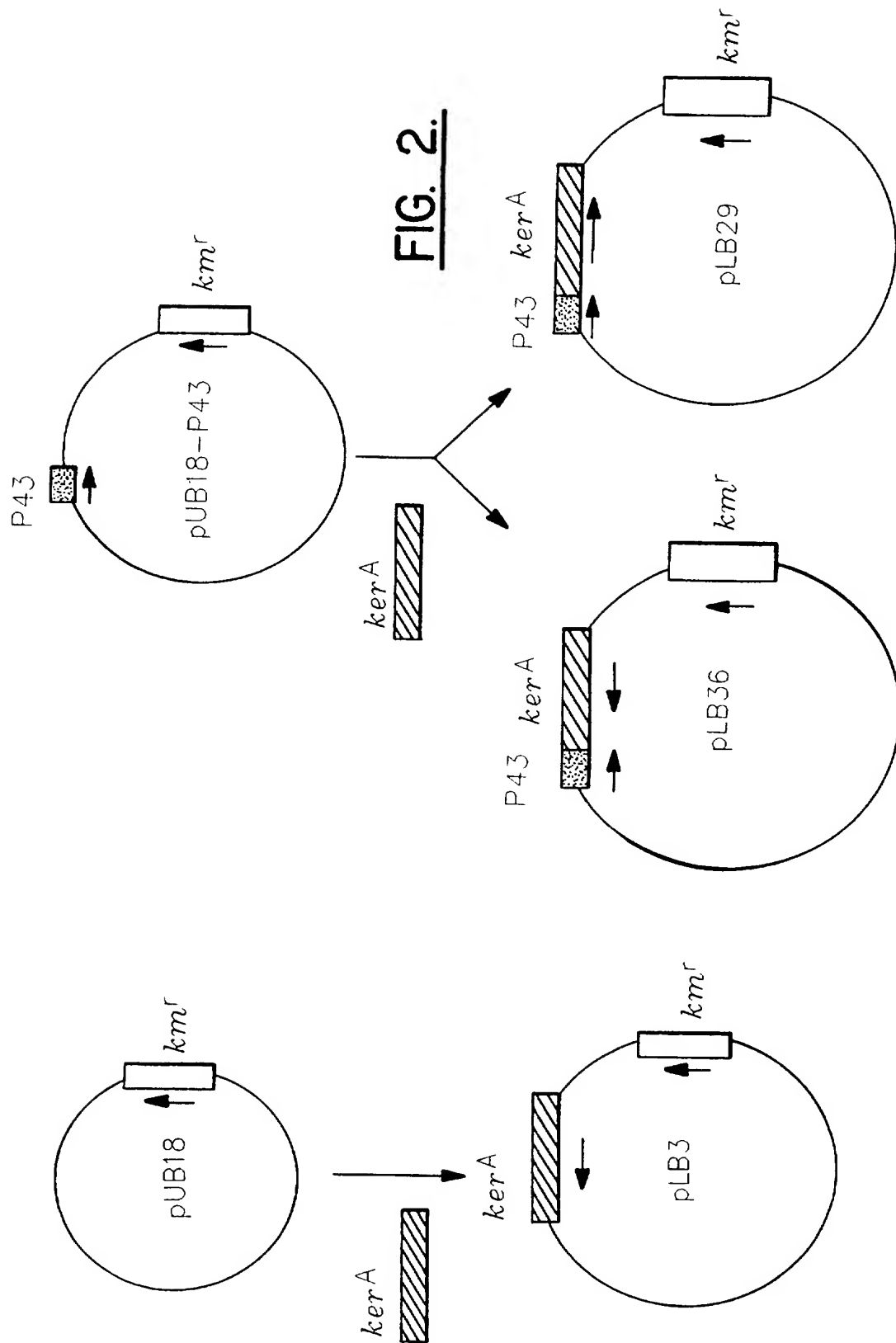


FIG. 1.

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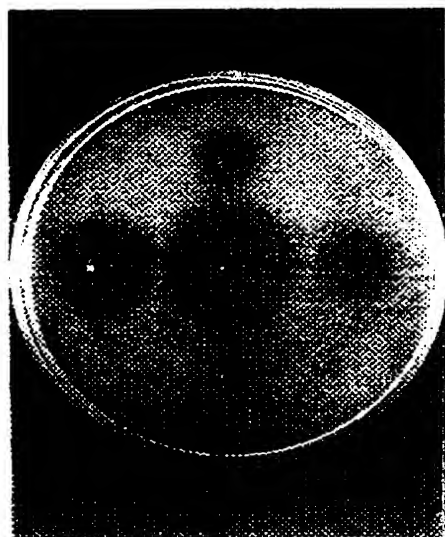


FIG. 3A

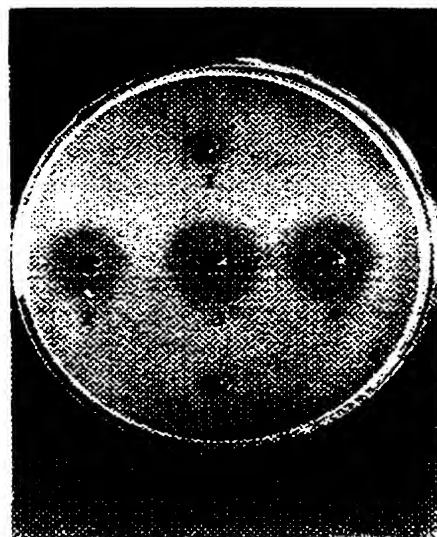
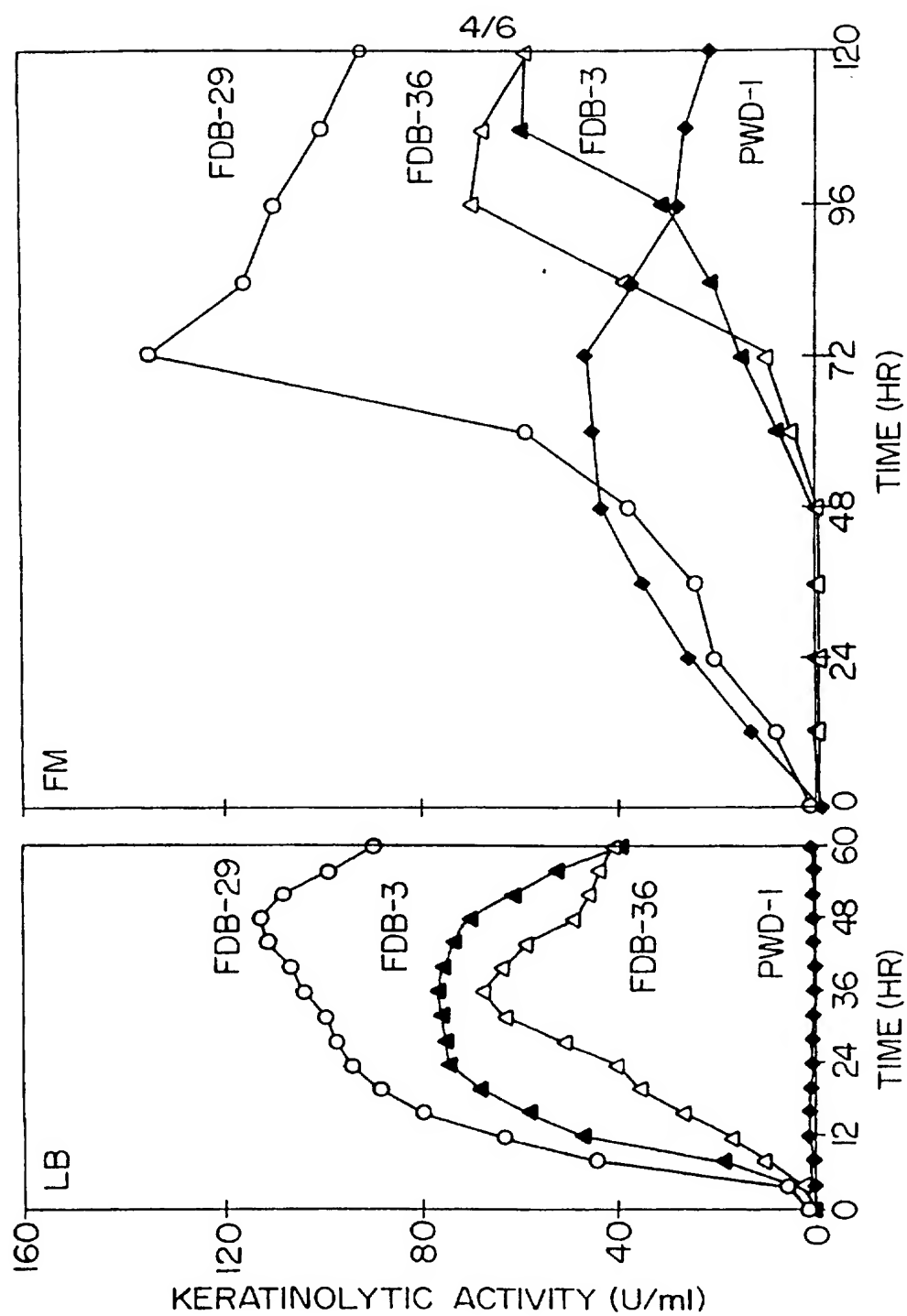


FIG. 3B

FIG. 4.

5 / 6

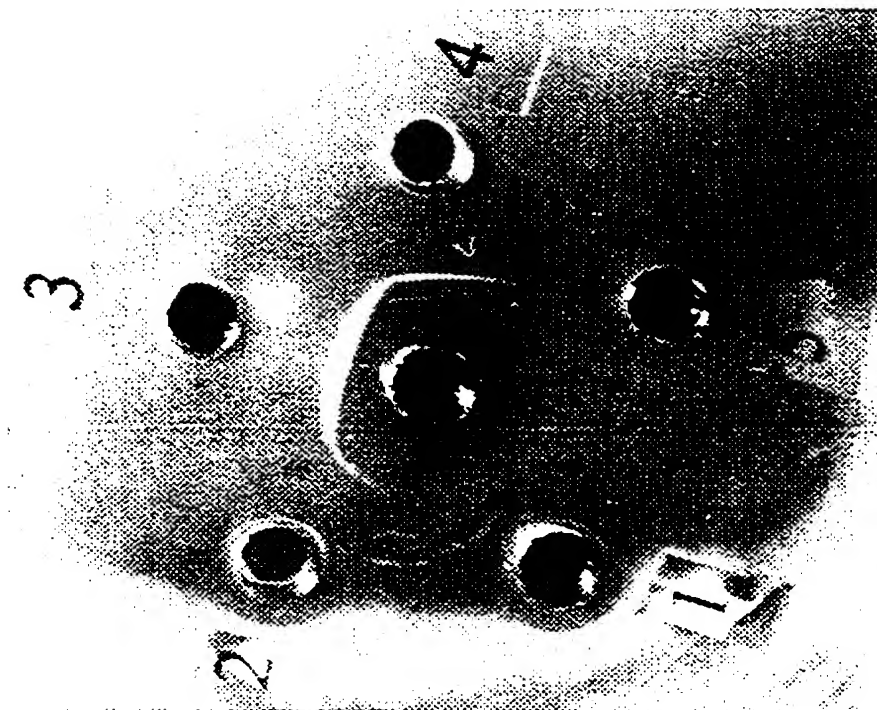


FIG. 5B.

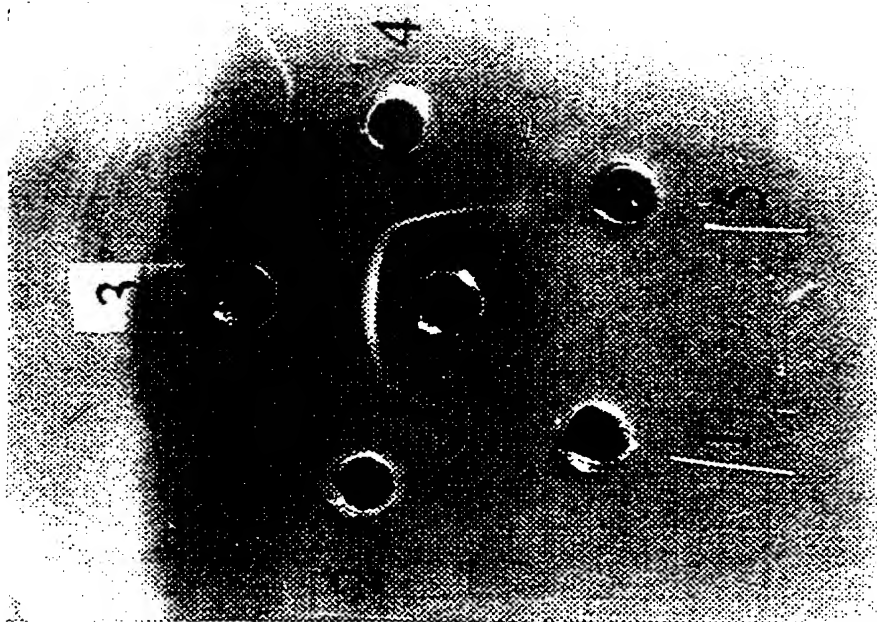


FIG. 5A.

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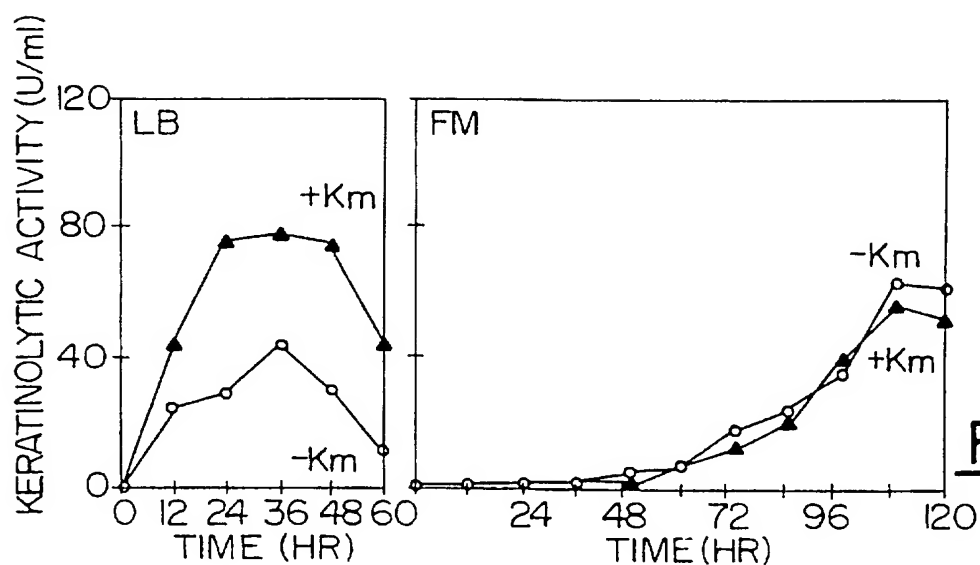


FIG. 6A.

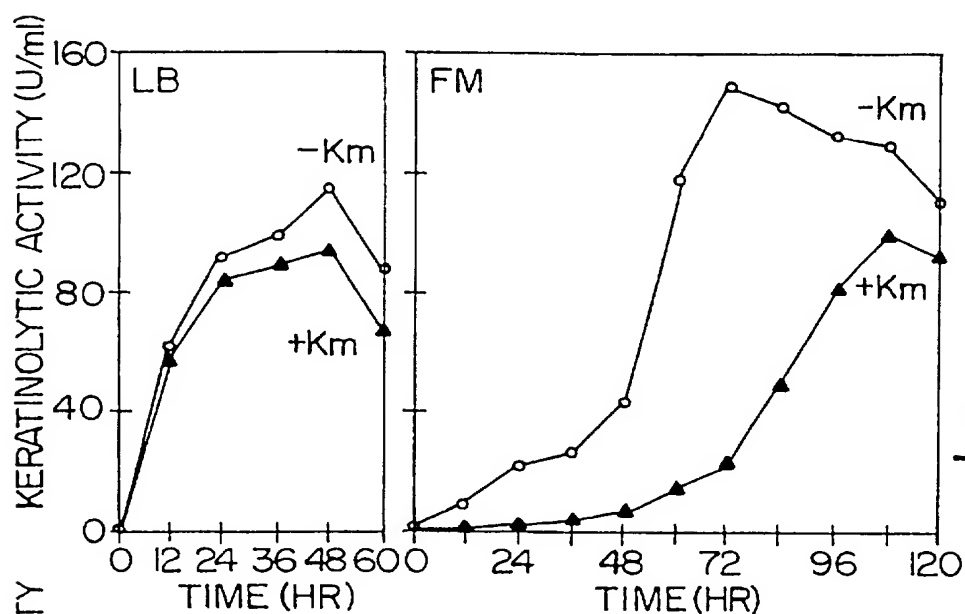


FIG. 6B.

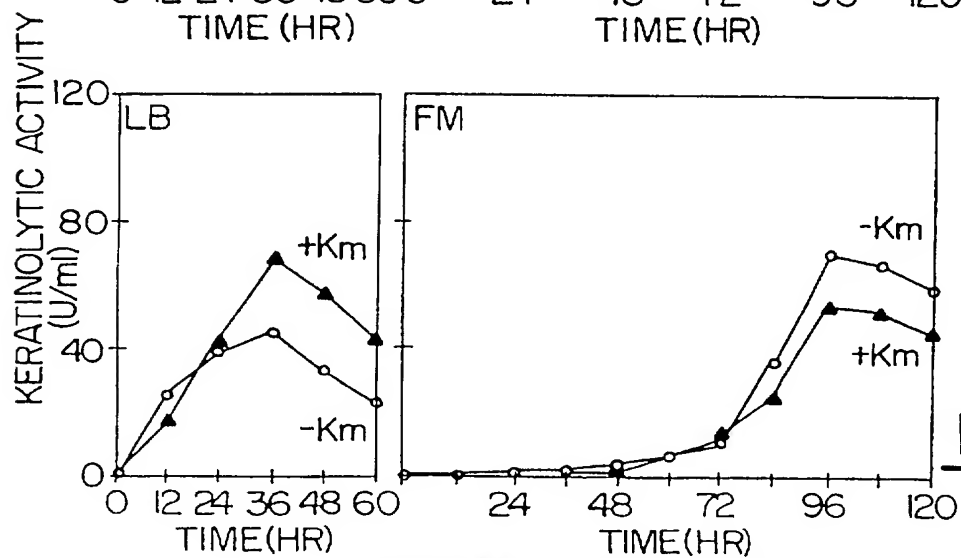
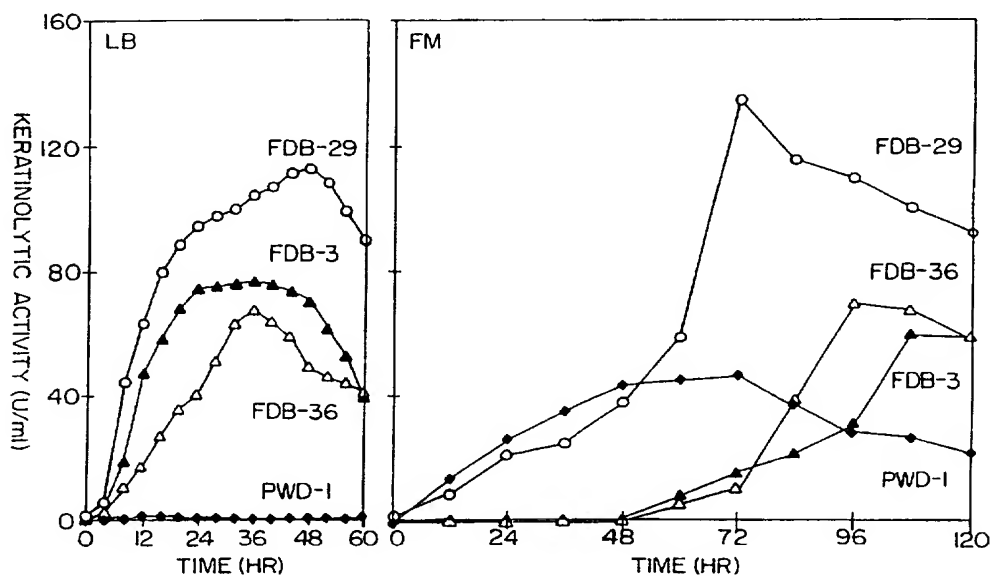


FIG. 6C.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/57, 1/21, 9/56, 15/75, 15/62 // (C12N 1/21, C12R 1:125)	A3	(11) International Publication Number: WO 97/39130 (43) International Publication Date: 23 October 1997 (23.10.97)
(21) International Application Number: PCT/US97/06477 (22) International Filing Date: 16 April 1997 (16.04.97) (30) Priority Data: 08/634,440 18 April 1996 (18.04.96) US (60) Parent Application or Grant (63) Related by Continuation US 08/634,440 (CON) Filed on 18 April 1996 (18.04.96) (71) Applicants (for all designated States except US): NORTH CAROLINA STATE UNIVERSITY [US/US]; 103 Holladay Hall, Campus Box 7003, Raleigh, NC 27695-7003 (US). UNIVERSITY TECHNOLOGIES INTERNATIONAL, INC. [CA/CA]; 609 14th Street, N.W., Calgary, Alberta (CA). (72) Inventors; and (75) Inventors/Applicants (for US only): SHIH, Jason, C., H. [US/US]; 100 Planters Wood Lane, Cary, NC 27511 (US). LIN, Xiang [CN/US]; Apartment 203, 3608 Horton Street, Raleigh, NC 27607 (US). WONG, Sui-Lam [CA/CA]; 38 Ranch Glen Drive, N.W., Calgary, Alberta T3G 1E3 (CA).	(74) Agents: SIBLEY, Kenneth, D. et al.; Myers, Bigel, Sibley & Sajovec, L.L.P., P.O. Box 37428, Raleigh, NC 27627 (US). (81) Designated States: AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), EE, EE (Utility model), ES, FI, FI (Utility model), GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 12 February 1998 (12.02.98)	

(54) Title: METHOD FOR EXPRESSING AND SECRETING KERATINASE**(57) Abstract**

The present invention provides a *Bacillus subtilis* host cell capable of expressing and secreting keratinase. The host cell contains a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith. The present invention also provides a method for producing keratinase enzyme. The method includes the steps of (a) culturing a *Bacillus subtilis* host cell containing a recombinant DNA molecule comprising vector DNA and DNA encoding *Bacillus licheniformis* PWD-1 keratinase enzyme operatively associated therewith, and (b) collecting keratinase enzyme from the cell culture.

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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 97/06477

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/57 C12N1/21 C12N9/56 C12N15/75 C12N15/62
 //(C12N1/21,C12R1:125)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 33056 A (NORTH CAROLINA STATE UNIVERSITY) 7 December 1995 cited in the application	1-5, 9-13, 16-19 6,14,20, 23-55
Y	see page 2, line 32 - page 4, line 3 see page 8, line 29 - page 11, line 24; figure 1	
X	WO 89 09278 A (NORTH CAROLINA STATE UNIVERSITY) 5 October 1989 see page 8, line 24 - page 11, line 33 -/--	1,3,5,9, 11,13

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

9 December 1997

Date of mailing of the international search report

18.12.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Montero Lopez, B

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/US 97/06477

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>PEI-ZHI WANG ET AL.: "Overlapping promoters transcribed by <i>Bacillus subtilis</i> sigma55 and sigma37 RNA polymerase holoenzymes during growth and stationary phases"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 259, no. 13, 10 July 1984, MD US, pages 8619-8625, XP002042749</p> <p>cited in the application</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	6,14,20
Y	<p>EP 0 310 137 A (ZYMOGENETICS, INC.) 5</p> <p>April 1989</p> <p>see the whole document</p> <p style="text-align: center;">---</p>	23-55
A	<p>XIANG LIN ET AL.: "Nucleotide sequence and expression of <i>kerA</i>, the gene encoding a keratinolytic protease of <i>Bacillus licheniformis</i> PWD-1"</p> <p>APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 4, April 1995, pages 1469-1474, XP002042752</p> <p>cited in the application</p> <p>see abstract</p> <p>see page 1471, right-hand column, paragraph 3 - page 1472, left-hand column, paragraph 1</p> <p>see page 1472, right-hand column, paragraph 2 - page 1473, right-hand column, paragraph 3; figure 9</p> <p style="text-align: center;">---</p>	1-22
A	<p>LIN X ET AL: "Gene sequence and expression of keratinase in the feather-degrading <i>Bacillus licheniformis</i> PWD -1."</p> <p>SIXTEENTH ANNUAL MEETING OF THE SOUTHERN POULTRY SCIENCE SOCIETY, ATLANTA, GEORGIA, USA, JANUARY 18-20, 1995. POULTRY SCIENCE 74 (SUPPL. 1). 1995. 201. ISSN: 0032-5791, XP002042753</p> <p>see the whole document</p> <p style="text-align: center;">-----</p>	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/06477

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/06477

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-22

Bacillus subtilis host cell containing recombinant DNA encoding Bacillus licheniformis PWD-1 keratinase enzyme; use thereof for producing and excreting the enzyme

2. Claims: 23-55

DNA molecule comprising DNA encoding kerA pre/pro processing and secretion region, and a heterologous DNA encoding an enzyme other than Bacillus licheniformis PWD-1 keratinase; Bacillus subtilis host cell containing the same; use thereof for producing and excreting such enzyme.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/06477

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9533056 A	07-12-95	AU 2472195 A	21-12-95
		EP 0764211 A	26-03-97
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